

CLAIMS

1. Method for preparing a lyophilized matrix which, upon contact with an aqueous carrier liquid and a gas, is reconstitutible into a suspension of gas-filled microbubbles stabilized predominantly by a phospholipid, said method comprising the steps of:
- 5 a) preparing an aqueous-organic emulsion comprising i) an aqueous medium including water, ii) an organic solvent substantially immiscible with water; iii) an emulsifying composition of amphiphilic materials comprising more than 50% by weight of a phospholipid and iv) a lyoprotecting agent;
- 10 b) lyophilizing said emulsified mixture, to obtain a lyophilized matrix comprising said phospholipid.
2. Method for preparing an injectable contrast agent comprising a liquid aqueous suspension of gas-filled microbubbles stabilized predominantly by a phospholipid, which comprises the steps of:
- 15 a) preparing an aqueous-organic emulsion comprising i) an aqueous medium including water, ii) an organic solvent substantially immiscible with water; iii) an emulsifying composition of amphiphilic materials comprising more than 50% by weight of a phospholipid and iv) a lyoprotecting agent;
- 20 b) lyophilizing said emulsion, to obtain a lyophilized matrix comprising said phospholipid;
- c) contacting said lyophilized matrix with a biocompatible gas;
- d) reconstituting said lyophilized matrix by dissolving it into a physiologically acceptable aqueous carrier liquid, to obtain a suspension of gas-filled microbubbles stabilized predominantly by said phospholipid.
- 25 3. Method according to claim 1 or 2 wherein the step a) of preparing the emulsion comprises the following steps:
- a1) preparing a suspension by dispersing the emulsifying composition and the lyoprotective agent in the aqueous medium;
- 30 a2) admixing the obtained suspension with the organic solvent;
- a3) submitting the mixture to controlled agitation, to obtain an emulsion.
4. Method according to any of the preceding claims, wherein the organic solvent has a solubility in water of less than 10 g/l.
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5. Method according to any of the preceding claims, wherein the organic solvent has a solubility in water of 1.0 g/l or lower.
6. Method according to any of the preceding claims, wherein the organic solvent has a solubility in water of 0.2 g/l or lower.
7. Method according to any of the preceding claims, wherein the organic solvent has a solubility in water of about 0.01 g/l or lower.
8. Method according to any of the preceding claims, wherein the organic solvent has a solubility in water of 0.001 g/l or lower.
9. Method according to claim 1, wherein the organic solvent is selected among branched or linear alkanes, alkenes, cyclo-alkanes, aromatic hydrocarbons, alkyl ethers, ketones, halogenated hydrocarbons, perfluorinated hydrocarbons and mixtures thereof.
10. Method according to claim 9 wherein the solvent is selected among pentane, hexane, heptane, octane, nonane, decane, 1-pentene, 2-pentene, 1-octene, cyclopentane, cyclohexane, cyclooctane, 1-methyl-cyclohexane, benzene, toluene, ethylbenzene, 1,2-dimethylbenzene, 1,3-dimethylbenzene, di-butyl ether and di-isopropylketone, chloroform, carbon tetrachloride, 2-chloro-1-(difluoromethoxy)-1,1,2-trifluoroethane (enflurane), 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane (isoflurane), tetrachloro-1,1,1,2-tetrafluoroethane, perfluoropentane, perfluorohexane, perfluoroheptane, perfluorononane, perfluorobenzene, perfluorodecalin, methylperfluorobutylether, methylperfluoroisobutylether, ethylperfluorobutylether, ethylperfluoroisobutylether and mixtures thereof
11. Method according to any of the preceding claims, wherein the amount of organic solvent is from about 1% to about 50% by volume with respect to the amount water.
12. Method according to any of the preceding claims wherein the lyoprotecting agent is selected among carbohydrates, sugar alcohols, polyglycols and mixtures thereof.

13. Method according to claim 12 wherein the lyoprotecting agent is selected among glucose, galactose, fructose, sucrose, trehalose, maltose, lactose, amylose, amylopectin, cyclodextrins, dextran, inuline, soluble starch, hydroxyethyl starch (HES), erythritol, mannitol, sorbitol, polyethyleneglycols and mixtures thereof.

14. Method according to claim 12 or 13 wherein the amount of lyoprotecting agent from about 1% to about 25% by weight with respect to the weight of water.

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15. Method according to any of claims 1, 2 or 3 wherein the phospholipid is selected among dilauroyl-phosphatidylcholine (DLPC), dimyristoyl-phosphatidylcholine (DMPC), dipalmitoyl-phosphatidylcholine (DPPC), diarachidoyl-phosphatidylcholine (DAPC), distearoyl-phosphatidylcholine (DSPC), dioleoyl-phosphatidylcholine (DOPC), 1,2 Distearoyl-sn-glycero-3-Ethylphosphocholine (Ethyl-DSPC), dipentadecanoyl-phosphatidylcholine (DPDPC), 1-myristoyl-2-palmitoyl-phosphatidylcholine (MPPC), 1-palmitoyl-2-myristoyl-phosphatidylcholine (PMPC), 1-palmitoyl-2-stearoyl-phosphatidylcholine (PSPC), 1-stearoyl-2-palmitoyl-phosphatidylcholine (SPPC),), 1-palmitoyl-2-oleylphosphatidylcholine (POPC), 1-oleyl-2-palmitoyl-phosphatidylcholine (OPPC), dilauroyl-phosphatidylglycerol (DLPG) and its alkali metal salts, diarachidoylphosphatidyl-glycerol (DAPG) and its alkali metal salts, dimyristoylphosphatidylglycerol (DMPG) and its alkali metal salts, dipalmitoylphosphatidylglycerol (DPPG) and its alkali metal salts, distearoylphosphatidylglycerol (DSPG) and its alkali metal salts, dioleoyl-phosphatidylglycerol (DOPG) and its alkali metal salts, dimyristoyl phosphatidic acid (DMPA) and its alkali metal salts, dipalmitoyl phosphatidic acid (DPPA) and its alkali metal salts, distearoyl phosphatidic acid (DSPA), diarachidoylphosphatidic acid (DAPA) and its alkali metal salts, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE), distearoyl phosphatidyl-ethanolamine (DSPE), dioleylphosphatidyl-ethanolamine (DOPE), diarachidoylphosphatidylethanolamine (DAPE), dillinoleylphosphatidylethanolamine (DLPE), polyethyleneglycol modified dimyristoyl-phosphatidylethanolamine (DMPE-PEG), polyethyleneglycol modified dipalmitoylphosphatidylethanolamine (DPPE-PEG), polyethyleneglycol modified distearoyl phosphatidyl-ethanolamine (DSPE-PEG), polyethyleneglycol modified dioleylphosphatidyl-ethanolamine (DOPE-PEG), polyethyleneglycol modified diarachidoylphosphatidylethanolamine (DAPE-PEG),

polyethyleneglycol modified dilinoleylphosphatidylethanolamine (DLPE-PEG), dimyristoyl phosphatidylserine (DMPS), diarachidoyl phosphatidylserine (DAPS), dipalmitoyl phosphatidylserine (DPPS), distearoylphosphatidylserine (DSPS), dioleoylphosphatidylserine (DOPS), dipalmitoyl sphingomyelin (DPSP),
 5 and distearoylsphingomyelin (DSSP) and mixtures thereof.

16. Method according to claim 1 wherein the emulsifying composition of amphiphilic materials comprises a phospholipid or an amphiphilic material bearing an overall net charge.

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17. Method according to claim 1, 2, 3 or 13, wherein the amount of phospholipid is from about 0.005% to about 1.0% by weight with respect to the total weight of the emulsified mixture.

18. Method according to claim 17 wherein the amount of phospholipid is from 0.01% to 1.0% by weight with respect to the total weight of the emulsified mixture.

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19. Method according to claim 1, 2 or 3 wherein the phospholipid includes a targeting ligand or a protective reactive group capable of reacting with a targeting ligand.

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20. Method according to any of claims 1, 2, 3, 15 or 16 wherein the emulsion further contains an amphiphilic material selected from lysolipids; fatty acids and their respective salts with alkali or alkali metals; lipids bearing polymers; lipids bearing sulfonated mono- di-, oligo- or polysaccharides; lipids with ether or ester-linked fatty acids; polymerized lipids; diacetyl phosphate; dicetyl phosphate; stearylamine; ceramides; polyoxyethylene fatty acid esters; polyoxyethylene fatty alcohols; polyoxyethylene fatty alcohol ethers; polyoxyethylated sorbitan fatty acid esters; glycerol polyethylene glycol ricinoleate; ethoxylated soybean sterols; ethoxylated castor oil; ethylene oxide (EO) and propylene oxide (PO) block copolymers; sterol esters of sugar acids; esters of sugars with aliphatic acids; esters of glycerol with (C₁₂-C₂₄) dicarboxylic fatty acids and their respective salts with alkali or alkali-metal salts; saponins; long chain (C₁₂-C₂₄) alcohols; 6-(5-cholesten-3 β -yloxy)-1-thio- β -D-galactopyranoside; digalactosyldiglyceride; 6-(5-cholesten-3 β -yloxy)hexyl-6-amino-6-deoxy-1-thio- β -D-galactopyranoside; 6-(5-cholesten-3 β -yloxy)hexyl-6-amino-6-deoxyl-1-thio- β -D-mannopyranoside; 12-(((7'-

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- diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoic acid; N-[12-
(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoyl]-2-
aminopalmitic acid; N-succinyldioleoylphosphatidylethanolamine; 1-hexadecyl-
2-palmitoylglycerophosphoethanolamine; palmitoylhomocysteine;
5 alkylammonium salts comprising at least one (C₁₀-C₂₀) alkyl chain; tertiary or
quaternary ammonium salts comprising at least one (C₁₀-C₂₀) acyl chain linked
to the N-atom through a (C₃-C₆) alkylene bridge; and mixtures or
combinations thereof.
- 10 **21.** Method according to claim 1 or 2 wherein the aqueous-organic emulsion of
step a) is subjected to a washing step before the lyophilizing step b).
- 22.** Method according to claim 1 or 2 wherein the aqueous-organic emulsion of
step a) is subjected to a microfiltration step before the lyophilizing step b).
- 15 **23.** Method according to claim 1 or 2 which further comprises adding an
aqueous suspension comprising a further amphiphilic compound to the
aqueous-organic emulsion obtained according to step a), before the
lyophilization step b), thus obtaining a second aqueous-organic emulsion
20 comprising said further amphiphilic compound.
- 24.** Method according to claim 23 which further comprises heating the mixture
of said aqueous suspension and of said aqueous-organic emulsion.
- 25 **25.** Method according to claim 23 wherein said mixture is heated at a
temperature of from about 40°C to about 80°C.
- 26.** Method according to claim 23 wherein said amphiphilic compound is a PEG-
modified phospholipid, a PEG-modified phospholipid bearing a reactive moiety
30 or a PEG-modified phospholipid bearing a targeting ligand
- 27.** Method according to claim 1, 2 or 23 which further comprises, before the
lyophilization step b), subjecting the aqueous-organic emulsion to a controlled
heating.
- 35 **28.** Method according to claim 27, wherein said controlled heating is effected at
a temperature of from about 60°C to 125°C.

29. Method according to claim 28, wherein said controlled heating is effected at a temperature of from about 80°C to 120°C.
30. Method according to claim 28, wherein said emulsion is contained in a sealed vial.
31. Method according to claim 2 or 3 wherein the biocompatible gas is selected among air; nitrogen; oxygen; carbon dioxide; hydrogen; nitrous oxide; inert gases; a low molecular weight hydrocarbon, including a (C₁-C₇) alkane, a (C₄-C₇) cycloalkane, a (C₂-C₇) alkene and a (C₂-C₇) alkyne; an ether; a ketone; an ester; a halogenated (C₁-C₇) hydrocarbon, ketone or ether; or a mixture of any of the foregoing.
32. Method according to claim 31 wherein the halogenated hydrocarbon gas is selected among bromochlorodifluoro-methane, chlorodifluoromethane, dichlorodifluoro-methane, bromotrifluoromethane, chlorotrifluoromethane, chloropentafluoroethane, dichlorotetrafluoroethane and mixtures thereof.
33. Method according to claim 31 wherein the halogenated hydrocarbon gas is a perfluorinated hydrocarbon.
34. Method according to claim 33 wherein the perfluorinated hydrocarbon gas is perfluoromethane, perfluoroethane, a perfluoropropane, a perfluorobutane, a perfluoropentane, a perfluorohexane, a perfluoroheptane; perfluoropropene, a perfluorobutene, perfluorobutadiene, perfluorobut-2-yne, perfluorocyclobutane, perfluoromethylcyclobutane, a perfluorodimethylcyclobutane, a perfluorotrimethylcyclobutane, perfluorocyclopentane, perfluoromethylcyclopentane, a perfluorodimethylcyclopentane, perfluorocyclohexane, perfluoromethylcyclohexane, perfluoromethylcyclohexane and mixtures thereof.
35. Injectable aqueous suspension of microbubbles filled with a biocompatible gas and comprising a stabilizing layer predominantly comprising a phospholipid, wherein said microbubbles have a number mean diameter (D_N) of less than 1.70 μm and a volume median diameter (D_{V50}) such that the D_{V50}/D_N ratio is of about 2.00 or lower.

36. Aqueous suspension according to claim 35 wherein said microbubbles have a D_N value of 1.60 μm or lower, preferably of 1.50 μm or lower, more preferably of 1.30 μm or lower.
- 5 37. Aqueous suspension according to claim 35 wherein said microbubbles have a D_{V50}/D_N ratio of about 1.80 or lower, preferably of about 1.60 or lower, more preferably of about 1.50 or lower.
- 10 38. Contrast agent for use in diagnostic imaging comprising an aqueous suspension according to any of the claims 35 to 37.
- 15 39. Method for diagnostic imaging comprising administering to a subject a contrast-enhancing amount of an aqueous suspension according to any of the claims 35 to 37 and imaging at least a part of said subject.
- 20 40. Method according to claim 39 which includes insonating said subject by means of an ultrasound device generating an ultrasound wave with a predetermined transmit frequency, from which a corresponding resonance size of microbubbles is determined, and administering a contrast agent comprising gas-filled microbubbles having a narrow size distribution and a mean size close to half the resonance size.